A New Future for Cassava in Asia:
Its Use as Food, Feed and Fuel to Benefit the Poor

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HIGH-VALUE CASSAVA: FROM A DREAM TO A CONCRETE REALITY

H. Ceballos, T. Sanchez[1], A. Rosero, A.P. Tofiño[2], K. Denyer, A. Smith[3], D. Dufour[4] N. Morante[1], J.C. Pérez[1], M. Fregene[1] and C. Egesi[1]

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is an important food security crop for many tropical and subtropical countries. It is also acquiring an increasing role in rural development as raw material for different industries. The most important industrial uses of cassava are as a source of energy in the feed industry, the bio-ethanol and starch industries, and for processed food. For cassava to be a suitable raw material for different industrial pathways, it has to have a competitive price, which is dependent on high and stable fresh root production, high dry matter content and adequate cultural practices that will maximize yields and reduce production costs. For years many institutions have successfully satisfied these needs. However, to consolidate and expand the industrial uses of cassava, the cassava breeding project at CIAT began increasing the emphasis in the search for value-added traits with the turn of the millennium. Several strategies have been implemented simultaneously. For the feed industry, the main objective is enhanced nutritional quality, particularly with regard to protein and pro-vitamin A carotenoids content. For the starch industry, amylose-free and high-amylose mutations have been identified. Inbreeding has been introduced to cassava genetic improvement because it offers many advantages, including a facilitated identification of useful recessive traits including new plant architecture types. Ongoing research for the production of doubled-haploid lines will reduce the time required to reach full homozygosity. Finally, CIAT has set up a high capacity root-quality laboratory to routinely screen the roots of the thousands of new genotypes generated every year.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial crop native to tropical America, with the probable center of origin in the southern rim of the Amazon basin of Brazil (Olsen and Schaal, 2001). Cassava is one of the most important sources of food energy in many tropical and subtropical countries. There are an estimated 200 million people who obtain more that 500 cal/day from cassava (Cock, 1985; Kawano *et al.*, 1998). The crop produces reasonably well under marginal conditions of climate and soil, and is frequently identified as a famine reserve due to its tolerance to drought and infertile soils and its ability to recover from disease and pest attacks. It can also produce competitively in non-marginal areas. Cassava offers the advantage of a flexible harvesting date, allowing farmers to hold the roots in the ground until needed (Iglesias *et al.*, 1997).

In addition to its important role in subsistence farming and food security, cassava is acquiring an increased role in rural development as raw material for many industrial applications for the production of animal feed, starch, bioethanol and processed food. The main strategy used until now to promote cassava as an industrial raw material has been to increase its productivity and/or reduce production costs, allowing for a competitive price of the roots. Productivity can be increased by genetic improvement to produce varieties with

[1]Cassava Breeding Project, International Center for Tropical Agriculture (CIAT), Cali, Colombia
[2] National University of Colombia, Palmira campus, Palmira, Colombia
[3] John Innes Centre, Norwich Research Park, Norwich, Norfolk, UK
[4] CIRAD-PERSYST, Montpellier, France

high and stable fresh root yields, with tolerance or resistance to the most important pests and diseases as well as with adaptation to abiotic stresses. A key trait for most industries is dry matter content, which has been successfully increased over the years (Kawano, 2003). Productivity can also be increased through adequate cultural practices in which proper fertilization and the control of weeds play a key role. The use of agents for the biological control of pests and diseases is an efficient approach, both from the economic and environmental points of view. Mechanization of planting and harvesting has proved to be effective in reducing production costs.

There has been, however, very little effort made to increase the value of cassava roots. The price of dried cassava roots, when used for the feed industry, is lower than that of maize because of their low levels of proteins. High carotenoids in the root is fundamental for cassava in human nutrition, but also has been proven to be advantageous in the production of animal feed (Posada *et al.*, 2006). In addition, cassava has the disadvantage of low genetic variability for starch quality traits. Compared with the many economically useful mutations found, for example, in the maize kernel (sweet corn, pop corn, waxy maize starch, opaque 2, amylose extender, etc.), very little variability has been reported for cassava. It is valid to assume that such variability exists in the crop, but have not yet been found; to identify them, new and different approaches would be required.

In spite of the problems mentioned above, the globalization of economies and new technological breakthroughs are offering a unique opportunity for cassava never available to the crop before. Tropical production of maize is facing increasing problems in competition with maize from temperate regions. This situation has prompted government and private sectors of many tropical countries to turn to cassava as a competitive alternative to imported maize. In addition, advances in molecular biology, genetic engineering, plant-tissue culture protocols and starch technologies provide important tools that will allow bridging the main gaps between cassava and the cereals.

This paper describes the approaches taken at CIAT to develop and identify cassava clones with higher value in their roots for different industries, and briefly describes some of the new high-value traits that have already been identified.

New Approaches for the Identification or Induction of High Value Traits

Cassava is a highly heterozygous crop. Most loci are, therefore, in a heterozygous status. Many high-value traits affecting, for example, the quality of starch are usually recessive. The fact that cassava seldom undergoes inbreeding drastically reduces the chance of [expectedly] low-frequency recessive alleles from expressing phenotypically. In addition, starch mutations in the roots are more difficult to detect than in grain kernels (where they can be easily identified by visual inspection without the need for any sophisticated tests). To detect a starch mutation in the cassava root, the breeder has to cut the roots and most likely needs to conduct a particular test (e.g. iodine test) or carry out biochemical analyses to be able to pick potentially useful variants. It is possible, therefore, that clones with valuable traits had already been grown in breeding nurseries but could not be detected and, not showing an outstanding agronomic performance, they were unfortunately discarded. To identify high-value traits affecting the quality of the root, it was necessary to implement

different breeding strategies to allow the expression of useful alleles and set up the proper screening protocols. The fact that roots are not reproductive or multiplicative organs may offer cassava (and other root crops) an advantage over the true seed-propagated crops. It is valid to assume that cassava roots could withstand mutations that would otherwise be lethal for reproductive organs such as the kernels of cereals.

Protocols for Systematic Characterization of Root Quality Traits

As stated above, one of the problems to identify high-value traits in cassava roots is that it is not easy to visually identify these traits. Therefore, a high-throughput laboratory was set up to be able to process roots from a large number of cassava genotypes each year. A brief description of the protocols developed and used at CIAT is provided in **Appendix 1**.

A second requirement for the development and identification of high value traits relates to the genetic approaches many of which were already described by Ceballos *et al.* (2006a.) The strategies that have been implemented for the development and identification of high-value traits in cassava are briefly described below.

Root Harvest, Handling and Evaluation

The analysis of reaction to post-harvest physiological deterioration (PPD) was conducted on several different types of genotypes. The harvest was done manually (as it is typically done) and with special care not to cause any injury to the roots. It is well known that rough handling creates localized traumas to the root tissue that accelerates PPD. Commercially sized roots were selected and placed in shelves under a roof but without walls. Air, therefore, circulated freely through the shelves. Evaluations were made at 5, 10, 20 and 40 days after harvest. Up to ten roots per genotype were evaluated at each evaluation date. The standard method for quantifying PPD (Wheatley *et al.*, 1985) removes the proximal and distal ends of the root to accelerate it. In this experiment, however, rather than using the standard approach, the roots were left untouched. This approach reduces the overall levels of PPD compared with previous reports (Sánchez *et al.*, 2005; Van Oirschot *et al.*, 2000), but allows a more realistic appraisal of the value of the tolerance detected. In other words, roots were stored as farmers or processors would keep them until processing.

Scoring the reaction to PPD is a destructive process. Seven transversal slices were cut along the root, starting at the proximal end. A score ranging from 1 to 10 was assigned to each slice, corresponding to the percentage of the cut surface showing discoloration (1=10%, 2=20%, etc). The mean PPD score for each root was calculated by averaging the scores of the seven transversal sections (Wheatley *et al.*, 1985). Roots showing symptoms of microbial rotting (very different from those related to PPD) or affected by insects, were not used for quantification of PPD.

Screening the Cassava Collection to Characterize Starch and Other Root Traits.

Currently CIAT is finalizing the screening of the entire cassava collection (with starch samples from around 6,000 landraces and improved germplasm (Sánchez *et al.*, 2009). Different tests were conducted following standard procedures (Aristizábal and Sánchez, 2007) and have been described above. Accessions from the germplasm collection were recovered from their *in-vitro* status, hardened and transplanted to the field. At the proper age

(10-12 months after planting) the plants grown in the field from each accession were harvested individually and their roots screened for different characteristics, including their starch biochemical and functional properties.

Table 1 summarizes the results of the most relevant root quality traits. Average dry matter content was 33.6%, with a range of variation from 14.28 to 48.12%. There was a slight asymmetry in the frequency distribution with a tendency of longer tails to the left. Cyanogenic potential ranged widely from 14 to 3,274 ppm, with an average of around 327 ppm (dry weight basis). Distribution was highly asymmetrical with a long tale to the right. Total and reducing sugars also showed an asymmetrical distribution with longer right tales, particularly in the case of reducing sugars. Average starch content was 84.5% (dry weight basis), with a tendency of values to concentrate towards the higher values, which accentuated a similar tendency observed for dry matter content (Sánchez *et al.*, 2009).

Amylose content ranged from 15.2 to 26.5% with an average of 20.7% (**Table 2**). The average amylose content of 20.7% is a very robust estimate since it is based on such a large sample of genotypes. Distribution was practically normal with a very slight asymmetry towards a longer right tale. No more than 1.5% of the samples had amylose values below 17.5 or above 24.5%. Water solubility and swelling power showed a more asymmetrical distribution with longer right tales. Average paste clarity was 45.2% with a large variation ranging from 12.5 to 96.6% (**Table 2**).

Table 1. Root quality traits from more than 4000 cassava genotypes.

Parameter	Dry matter content (% fresh root)	Cyanogenic potential (ppm of DM)	Sugar content		Starch content (% of DM)
			Total (% of DM)	Reducing (% of DM)	
Maximum	48.1	3,274	18.8	15.7	91.0
Minimum	14.3	14	0.2	0.0	65.0
Average	33.6	327.4	3.8	1.3	84.5
St.Deviation	6.47	397.7	2.32	1.43	3.34
Skewness	-0.40	2.96	1.76	3.08	-0.65
No. of accessions	4,051	4,050	4,049	4,049	4,049

Source: *Sánchez et al., 2009.*

Table 2. Starch quality traits from more than 4,000 cassava genotypes.

Parameter	Amylose content (%)	Water solubility (% db)	Swelling power (% g/g)	Paste clarity (%)
Maximum	26.5	16.6	15.5	96.6
Minimum	15.2	0.2	0.8	12.5
Average	20.7	2.2	4.6	45.2
St. Dev.	1.61	1.59	2.31	10.54
Skewness	0.22	1.77	1.53	-0.30
No. of accessions	4,042	4,050	4,050	4,044

Source: *Sánchez et al., 2009.*

Table 3. Pasting properties from starches of more than 4000 cassava genotypes.

Parameter	Pasting temperature (°C)	Maximum viscosity (cP)	Break-down (cP)	Consistency (cP)	Setback (cP)	Ease of cooking (min)
Maximum	71.2	1505.0	859.0	626.0	273.0	5.6
Minimum	58.8	146.0	28.1	0.0	-702.0	1.1
Average	65.3	777.5	298.1	155.8	-144.5	2.8
St.Dev.	1.75	165.03	107.1	57.8	96.2	0.72
Skewness	-0.13	0.22	0.81	0.94	-0.38	0.33
No. of accessions	4,051	4,051	4,051	4051	4,051	4,051

Source: *Sánchez et al., 2009.*

Pasting properties of the samples analyzed are described in **Table 3.** Average pasting temperature was 65.3°C and ranged from 58.8 to 71.2. Distribution frequency was relatively symmetrical as for the other parameters described in **Table 4**. Maximum viscosity averaged at 777.5 cP, with a wide range of variation from 146 up to 1505 cP. Breakdown ranged from 28.1 to 859.0 cP with an average around 298.1 cP. Consistency ranged from 626 down to 0 cP with an average of 155.8 cP. Finally, average setback was -144.5 cP with a minimum of -702.0 and a maximum observed value of 273.0 cP.

The results presented in Sánchez *et al.* (2009) article were exploratory. In most cases, samples were un-replicated. However when unusual data were observed, because of the particular interest in identifying unusual starch types, it prompted a second analysis to confirm the data. This approach, although it is limited from the experimental point of view, allowed the analysis of such a large number of genotypes, and provides very reliable information since at least outlaying data points were confirmed. One problem that remains unsolved is the possibility of a plant, for unknown reasons, producing starch samples with characteristics that may not be representative of that genotype. By and large, however, average values presented in this study should be very robust and properly represents starch characteristics of cassava. The range of variation is also useful to provide an idea of what traits may offer alternatives for further genetic improvement.

For some traits (water solubility, swelling power, paste clarity, paste breakdown, consistency, and setback) there was a large range of variation. It is very interesting to examine the relationships of these attributes from data of all evaluated clones (e.g. breakdown versus swelling power). More investigation and comparison on starch structure of clones with a great difference in functionalities are of great interest and this is currently under way.

Introduction of Inbreeding in Cassava

The introduction of inbreeding in the genetic improvement of cassava offers several advantages which have been described (Ceballos *et al.*, 2004; Ceballos *et al.*, 2007a; Contreras Rojas *et al.*, 2009; Pérez *et al.*, 2005a; 2005b). An advantage which has a direct bearing with the theme of this article, is that it would allow for the identification of useful recessive traits (such as the starch quality mutants found in different crops), which may lead to the development of value-added genetic stocks.

CIAT – supported by the Rockefeller Foundation and in collaboration with cassava breeding programs in Africa, Asia and Latin America – began in 2004 a research project to improve tolerance to inbreeding in elite germplasm. Elite germplasm was self-pollinated to generate a large number of botanical seeds with varying degrees of inbreeding. The project also involved the development of a microspore culture protocol for the production of doubled haploids. As soon as partially inbred plants are produced, the search for useful starch and other high-value traits is initiated. The production of doubled haploids provides an appealing option for the introduction of inbreeding in cassava genetic improvement by drastically reducing the time required to produce homozygous parental lines. Two interesting results can be used to illustrate the relevance of this approach.

Amylose-free starch mutation

After several years in search of an amylose-free mutation, this characteristic was finally identified in the self-pollinated genotype AM 206-5 and reported in the literature the following year (Ceballos *et al.*, 2007b). This starch mutation had been requested by the starch industry for many years. In addition to the information already published in relation to this mutation, a comparison of normal and waxy starches from different crops has been conducted. **Tables 4-7** present the most relevant information in relation to this comparison.

Table 4. Physico-chemical properties of normal and waxy starches from different crops.

	Amylose content (%)	Paste Clarity (%)	λ Max
Normal starches			
Maize	19.9 (±0.4)	11 (±2.7)	590
Potato	27.7 (±0.5)	88 (±0.8)	591
CM 523-7 (Cassava)	19.8 (±1.3)	50 (±3.5)	593
MPer 183 (Cassava)	19.5 (±1.8)	51 (±3.8)	590
MTai 8 (Cassava)[1)]	16.5 (±0.6)	47 (±0.8)	592
Waxy starches			
Maize	0	42 (±1.1)	529
Potato	7.7 (±0.83)	92 (±1.4)	550
Cassava (AM 206-5)	0	61 (±0.7)	535

[1)] MTai 8 = Rayong 60

Identification of a new plant architecture mutation

One of the major changes that cassava research at CIAT has had in the past few years is the systematic introduction of partial inbreeding throughout the germplasm collection to allow for the expression (and therefore identification) of recessive traits. Most of these recessive traits result in undesirable characteristics as it happens in other crops, but few offer interesting advantages. During the May-2008 to April-2009 season, a new generation of self-pollinated germplasm was evaluated in the field. It was very apparent in one family composed from 16 genotypes that the parental progenitor (MVen 331) carried an unusual trait. Half of the 16 plants showed a distinctive feature: leaves without petioles. Moreover, several of these plants had a unique and distinctive phenotype (**Figure 1**) with absence of branching at least for the first 6-8 months of age.

Table 5. Pasting characteristics of normal and waxy starches from different crops.

Sample identification	Pasting temp. (°C)	Peak viscosity (cP)	Breakdown (cP)	Setback (cP)	Consistency (cP)
Native starches					
Maize	89.0 (±0.85)	176 (±4)	-30 (±4)	-15 (±3)	-45 (±6)
Potato	65.2 (±0.06)	2550(±15)	1204 (±29)	-1082 (±2)	108 (±5)
CM 523-7	63.3 (±0.12)	1006 (±14)	500 (±22)	-364 (±8)	137 (±14)
MPer 183	64.8 (±0.12)	979 (±12)	482 (±15)	-267 (±10)	215 (±8)
MTai 8	63.7 (±0.00)	876 (±13)	455 (±0)	-338 (±4)	117 (±4)
Waxy starches					
Maize	70.9 (±0.00)	973(±22)	307 (±25)	-289 (±4)	31 (±2)
Potato	65.9 (±0.12)	2491 (±49)	1287 (±30)	-1268	13(±3)
Cassava	67.4 (±0.00)	1119 (±11)	631 (±8)	-595 (±12)	37 (±4)

Table 6. Solubility and swelling values of normal and waxy starches from different crops. Analyses were made at three different final temperatures (60, 75 and 90°C)

Sample identification	Solubility (%db)			Swelling index (g/g)		
	60°C	75°C	90°C	60°C	75°C	90°C
Normal starches						
Maize	0,45 (±0.04)	2.66	10.51	2.76 (±0.16)	6.82 (±0.06)	12.2
Potato	2.5 (±0.66)	5.2	6.0 (±0.21)	13.8 (±0.06)	35.8 (±1.72)	51.1 (±2.92)
CM 523-7	3.1 (±0.27)	5.6	7.1 (±0.41)	22.9 (±1.42)	36.4 (±0.53)	42.4 (±2.62)
MPer 183	2.4 (±0.37)	5.5	7.3 (±0.28)	14.9 (±0.86)	34.5 (±3.3)	40.3 (±2.60)
MTai 8	2.3(±0.12)	5.3	7.6 (±0.07)	18.2 (±1.15)	37.2 (±2.87)	37.0 (±0.54)
Waxy starches						
Maize	0.74 (±0.04)	1.79	3.98	2.24 (±0.06)	36.13	38.34
Potato	0.4 (±0.15)	1.9	n.a.	11.4 (±0.67)	86.6 (±3.67)	n.a.
Cassava	0.6 (±0.07)	5.2	8.8 (±0.35)	4.5 (±0.67)	48.2 (±1.59)	54.7 (±1.63)

n.a. = not available because of difficulties in separations

The phenotype depicted in **Figure 1** offers interesting potential commercial applications. The most immediate one would be for the production of dried cassava foliage. One of the bottlenecks for this new market for cassava is the costs involved in harvesting the foliage. The only practical approach is a mechanical harvest that would also carry a considerable amount of young stems and petiole tissue. Since the kind of plants shown in **Figure 1** do not have petioles, nor any branching, at harvest of the leaves these could be easily stripped off the stem. The result would be a reduced cost of harvest, and, because the reduced proportion of petiole and young stems, a better quality of the foliage with a lower fiber content. This last characteristic would be fundamental for the use of dried foliage in the composition of diets for the poultry industry.

Table 7. Syneresis in normal and waxy starches from different crops after refrigreration or freezing for a period of up to five weeks.

Type of starch	Duration of storage of gels					
	0	1	2	3	4	5
	Syneresis (%) in refrigerated gels					
Normal cassava	0.0	0.0	1.2	3.7	6.7	8.5
Waxy cassava	0.0	0.0	0.0	0.0	0.0	0.0
Normal maize	5.0	8.3	14.0	25.0	35.0	42.0
Waxy maize	0.0	0.0	0.0	0.0	0.0	0.0
Normal potao	0.0	5.0	14.7	21.3	26.0	32.0
Waxy potato	0.0	2.0	2.0	2.0	4.0	6.0
	Syneresis (%) in frozen gels					
Normal cassava	0.0	0.0	8.0	11.0	14.7	21.7
Waxy cassava	0.0	0.0	0.0	0.0	0.0	0.0
Normal maize	11.3	15.3	19.7	26.3	31.7	36.3
Waxy maize	0.0	2.1	1.9	2.3	2.3	3.3
Normal potato	0.0	12.7	22.7	28.0	31.3	41.0
Waxy potato	0.0	4.2	6.0	6.7	7.7	7.7

Figure 1. Illustration of a plant type mutation resulting in leaves without petioles and a very erect architecture.

A second important potential application of this mutation would be the possibility of drastically increasing plant densities in commercial planting of cassava. It should also be easy to accept the idea that this new plant type could allow for higher plant densities in cassava fields. Perhaps as many as 30,000 plants per hectare could be used. This concept is important because most of the genetic gains achieved through the last century relates to modifications in plant architecture. The use of semi-dwarf wheat and rice varieties has led to the highly successful green revolution. In the case of maize, if there is a single characteristic that can explain the consistent gains observed after the first introduction of commercial hybrids, it is the reduced plant height with increased tolerance to higher plant densities (Troyer, 2006). Today, modern hybrid maize is planted at much higher densities than 40-50 years ago. So, this mutation observed in cassava may lead to a new plant type and, perhaps, a green revolution for this crop. It should be mentioned that the petioles mutation had been known in cassava for a long time. There are few accessions in the collection with this trait (in addition to the progenitor which does not have the mutation in homozygous condition, MVen 332 shows the petioles phenotype). What this mutation brings in is the certainty of the genetic nature of this mutation, the reasonable hypothesis that it is the result of a single recessive mutation and, more importantly, the combination of the petioles trait with short plant height and non-branching architecture.

Crosses have been or will be made among these mutant plants (in few of them there was a very late flowering) with those accessions in the germplasm collection that show the petioles trait to initiate a breeding with this gene pool characterized by the petioles, non-branching phenotype. These crosses will recover the vigor lost as a result of the inbreeding depression typical of S1 genotypes.

Mutagenesis and the "Tilling" System

Breeders have used chemical products or irradiation such as gamma rays to induce mutations and generate genetic variability with relative success, particularly in the decades of the 1950s and 1960s (Maluszynski *et al.*, 2001; Ahloowalia *et al.*, 2004). Mutation breeding has a few drawbacks. Events are totally random, recessive in nature and usually appear as chimeras. Therefore, thousands of genotypes need to be evaluated before a useful mutation in the desired gene can be found. With the advent of molecular biology tools, an interesting system was developed to overcome some of the limitations of mutation breeding. DNA TILLING (for *Targeted Induced Local Lesions in Genome*) has been successfully used in different plant species (McCallum *et al.*, 2000; Perry *et al.* 2003; Till *et al.* 2003). Sexual seeds are mutagenized and, to avoid ambiguities caused by chimeras in the first generation plants ($\mathbf{M_1}$), they are self-pollinated. The resulting plants ($\mathbf{M_2}$) are then evaluated while DNA is extracted from them. For screening purposes, DNAs are pooled eightfold to maximize the efficiency of mutation detection (description of the TILLING method adapted from Till *et al.*, 2003).

CIAT participated in a project led by Universidad Nacional de Colombia and supported by the IAEA (International Atomic Energy Agency). About 4,000 seeds from six different cassava clones were irradiated with gamma rays (using a Cobalt 60 source with a dosage level of 200 Gy) or with fast neutrons. Seeds were germinated and transplanted to the field early in 2004. Plants were carefully evaluated in search of promising mutant forms

(although it was recognized that the occurrence of chimeras and the lack of expression of recessive mutations would certainly reduce the probabilities of finding such mutants at the $\mathbf{M_1}$ stage). As soon as plants started to produce viable flowers, they were self-pollinated. As many as 5,000 $\mathbf{M_2}$ seeds, from about 140 different $\mathbf{M_1}$ plants, have been obtained. Several genes related to starch biosynthesis were targeted for TILLING analysis. Several mutations were identified in the $\mathbf{M_2}$ generation but only the two most interesting will be described herein.

Small granule – High amylose starch mutation

This mutation has been reported and described already in the literature (Ceballos *et al.*, 2008). The initial discovery was facilitated by the unusual starch granule size which is about 1/3 the normal size for cassava. **Figure 2** illustrates how different the starch granules of this mutation are, not only in relation to size but also regarding its surface. Normal cassava starch granules have a very smooth surface. However, the surface of the granules in the mutated genotype is very irregular and rough (**Figure 2**).

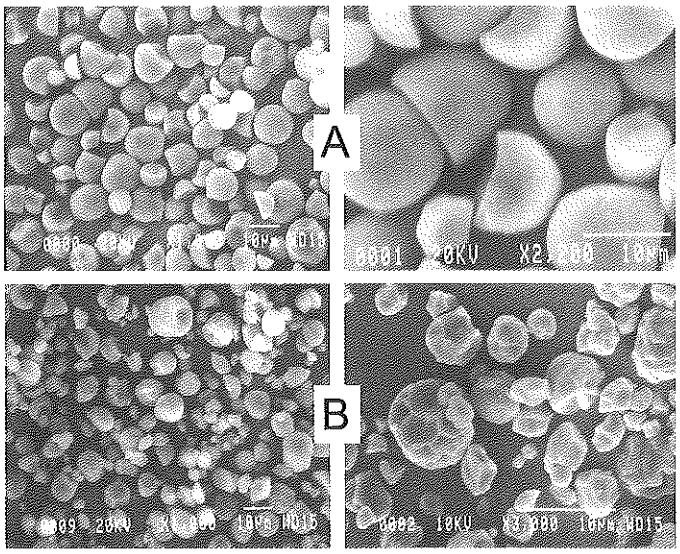

Figure 2. Scanning electron microscope photographs comparing normal (A) and mutant (B) cassava starch granules.
***Source**: Ceballos et al., 2008.*

The small size and irregular surface of the starch granules would make this mutation ideal for ethanol production because it facilitates the activity of starch degrading enzymes (Lehman and Robin, 2007; Thu *et al.*, 2007). The production of bio-ethanol from starch requires its degradation (liquefaction and saccharification), prior to the initiation of fermentation. However, the mutation also has a biochemical abnormality, with almost twice the normal levels of amylose (Ceballos *et al.*, 2008; Sánchez *et al.*, 2009). Amylose is more difficult to degrade (Sharma *et al.*, 2007). It is not possible at this time to know if the morphology of the starch granule will be more prevalent than the biochemical characteristics

(high amylose) of the mutation in the process of starch hydrolysis. When enough roots can be produced this analysis will be made.

The higher-than-normal level of amylose in these mutations has important commercial implications. Increased amylose levels leads to slowly digestible and resistant starches (Jobling, 2004; Lehman and Robin, 2007), which has a distinctive advantage in health, particularly in diabetes management. Slowly digestible starches may influence satiety and help control overweight problems, and they have also been linked to improved mental performances (Lehman and Robin, 2007). In addition, high-amylose starches in different crops offer advantages in the production of sweets, adhesives, corrugated boards and in the paper industry, and reduces the uptake of fat in certain fried products (Jobling, 2004). Very high levels of amylose result in "resistant" starches. Maize starches with more than 50% and up to 90% amylose can be produced commercially. Resistant starches cannot be digested but they are rather fermented in the large intestine, resulting in the production of butyrate that has been found to be beneficial to colon health (Jobling, 2004).

Tolerance to post-harvest physiological deterioration (PPD)

A second high-value trait that was identified in the mutagenized population was tolerance to PPD. Two genotypes were tentatively characterized as PPD-tolerant (2G15-1 and 5G108-8). Quantification of the reaction to PPD requires many commercial-size roots; these genotypes were first multiplied to produce the number of roots required. The result of this evaluation will be described below after other sources of tolerance have been described.

Rapid Cycling Recurrent Selection to Increase Carotenoids Content in Cassava Roots

The HarvestPlus initiative aims at reducing the problems derived from micronutrient deficiencies. The impact of vitamin A deficiency worldwide is an avoidable tragedy. The first step in the identification or production of cassava clones with enhanced nutritional value was the systematic and massive screening of landraces from CIAT's germplasm collection and key improved clones. The screening involved the evaluation for many micronutrients, but currently the work concentrates on pro-vitamin A carotenoids. Germplasm evaluated and the quantification methods were described by Chávez *et al.* (2005). Pro-vitamin A carotenoid levels have been found to be as high as 18 μg/g (fresh root basis) in the roots and considerably higher in the leaves. β-carotene has been found to be the most important component in these measurements. High carotene roots have a tendency for reduced or delayed post-harvest physiological deterioration (Sánchez *et al.*, 2005).

A normal recurrent selection cycle in cassava requires about eight years for completion (Morante *et al.*, 2005). This was considered to be too lengthy for a high-heritability trait as is the case of the carotenoids content in cassava roots. Therefore, a shorter cycle was implemented as illustrated in **Figure 3**. Each selection cycle did not go beyond the F1 stage of selection (when only one plant per genotype is available). Selected plants from the F1 nurseries were then planted in a new crossing block if they were late flowering (in this case each selection cycle required three years for completion). However, if plants flowered earlier, the cycle could be reduced to only two years. This implied making blind crosses among different genotypes without the certainty that they would be selected because of their high carotenoids contents.

Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8
Traditional recurrent selection scheme for cassava							
Planting crossing blocks	Seed from crosses harvested	F1 trial	Clonal evaluation trial	Preliminary yield trial	Advanced yield trial	Regional trial. 1st year.	Regional trial 2nd year
Rapid cycling recurrent selection for increased carotenoids content (late flowering clones)							
Planting crossing blocks	Seed from crosses harvested	F1 trial	Planting crossing blocks	Seed from crosses harvested	F1 trial		
Rapid cycling recurrent selection for increased carotenoids content (early floweringclones)							
Planting crossing blocks. Harvest of seed	F1 trial	Planting crossing blocks. Harvest of seed	F1 trial	Planting crossing blocks. Harvest of seed	F1 trial	Planting crossing blocks. Harvest of seed	F1 trial

Figure 3. Illustration of chronograms used in the rapid cycling recurrent selection approach to increase the carotenoids content in cassava roots.

Results from this approach were outstanding. **Figure 4** presents the gains from selection for high carotenoids content using the rapid cycling recurrent selection approach. The data presented are the results of the seedling (F1 trials) nurseries during the last three years of work. Every year an average of almost 3 µg carotenoids/g of fresh root could be added to the best genotypes. These are unprecedented gains for a crop like cassava. These results also highlight the importance of adapting the breeding schemes to the specific needs and characteristics of the trait to be improved.

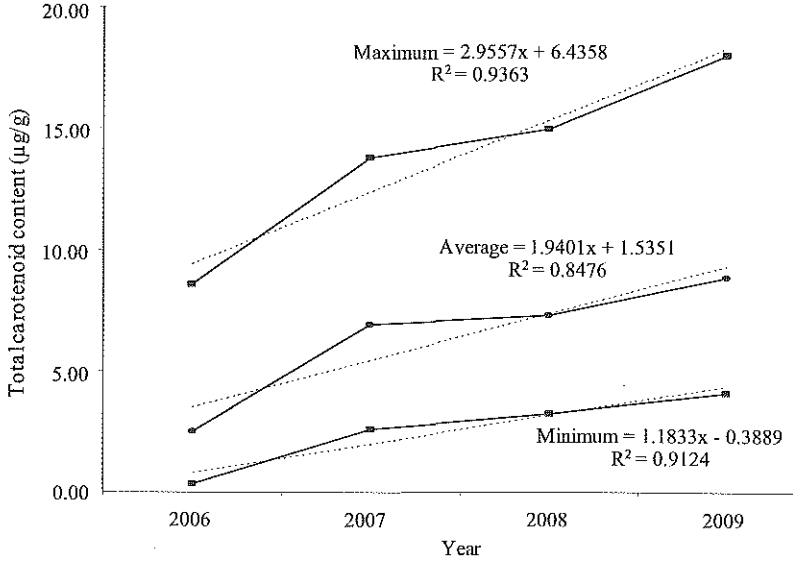

Figure 4. Gains from selection for high carotenoids content using the rapid cycling recurrent selection approach. Data presents the results of the seedling (F1 trials) nurseries during the last three years of work.

Interspecific Crosses

Tolerance to PPD

For several years CIAT has been working to introgress the tolerance to PPD found in the wild relative *Manihot walkerae* (Bertram, 1993; Cuambe, 2007; Fregene *et al.*, 2006). The interspecific cross was originally made with the elite genotype SM909-25 and then a first back-cross to SM909-25 was made to generate family BC289. In addition, the interspecific cross was also crossed to a different elite *M. esculenta* clone: MTai 8 (developed in Thailand and released with the name Rayong 60) to produce family BC284. Many genotypes were generated and evaluated in these two backcrosses (Cuambe, 2007) and three of them were selected for this study (BC284-42, BC284-49 and BC289-30). Results of this strategy are described later in this article.

Increased protein conten:

CIAT has also carried out a long-term project for higher protein content in the roots introgressed from accessions of wild relatives (*M. esculenta* subsp. *fabellifolia* and *M. tristis*) into cassava (CIAT, 2004). A total of 49 interspecific crosses having a range from 6.39 to 10.46% in protein content have been selected and back-crossed into the elite *M. esculenta* clone MTai 8 (Rayong 60 from Thailand). More than 6,000 back-crosses (BC) have been made. Further work is required to recover the root yield potential typical of *M. esculenta,* while maintaining (or even increasing) the current protein contents observed in the BC_1 populations. The issue of quality of proteins is also relevant and the profiling of amino acids is currently underway. In the process of screening for micronutrient content in cassava roots, a serendipitous discovery of interesting variation for crude protein content was made. Certain germplasm, like clone MCol 2436, has the advantage of a high crude protein content (around 7% on dry matter basis) combined with a higher carotene content (7.24 μg total carotenes/g fresh roots). A report on high protein cassava has already been published (Ceballos *et al.*, 2006b).

The standard methodology for estimating protein content has been traditionally through the indirect method of quantifying N by the Kjeldahl method and then multiplying it by the constant 6.25. However, it has been suggested that the N-to-protein conversion factor, in the case of cassava roots, may be considerably lower because of the presence of non-protein sources of N (Hock-Hin and Van-Den, 1996). Crosses have been made among the high-protein clones (based on the indirect Kjeldahl method) that had been identified. The resulting 393 genotypes were grown in Palmira (Colombia), and flour from their roots obtained. A direct method for the quantification of total soluble protein content (TSPC) was tested using the Bradford protein assay (Kruger, 1995; Bradford, 1976), which is a colorimetric method using the BioRad Dye Reagent (BioRad 500-0006). Three aliquots per sample were quantified. A group of 45 genotypes (selected to represent low, intermediate and high levels of TSPC) were also analyzed for their N content using the conventional Kjeldahl method.

Preliminary results confirmed a large variation in soluble proteins with a ten-fold difference between the high and low values (**Table 8).** However, the actual range of variation for TSPC was "compressed" compared with the variation based on indirect protein quantifications based on N contents (Kjeldahl's method).

Table 8. Variation in the total soluble protein contents of (TSPC) samples of 393 genotypes using the colorimetric Bradford protein assay. Three aliquots were taken per sample. Coefficient of variation for each individual sample was obtained and is presented in the right column. Reliability of the method is excellent with the average coefficient of variation below 6%.

Parameter	Soluble protein (%)	Coefficient of variation
Average	0.616144	5.848925
Max	1.02581	32.2906
Min	0.097302	0.102535
St Dev	0.132802	

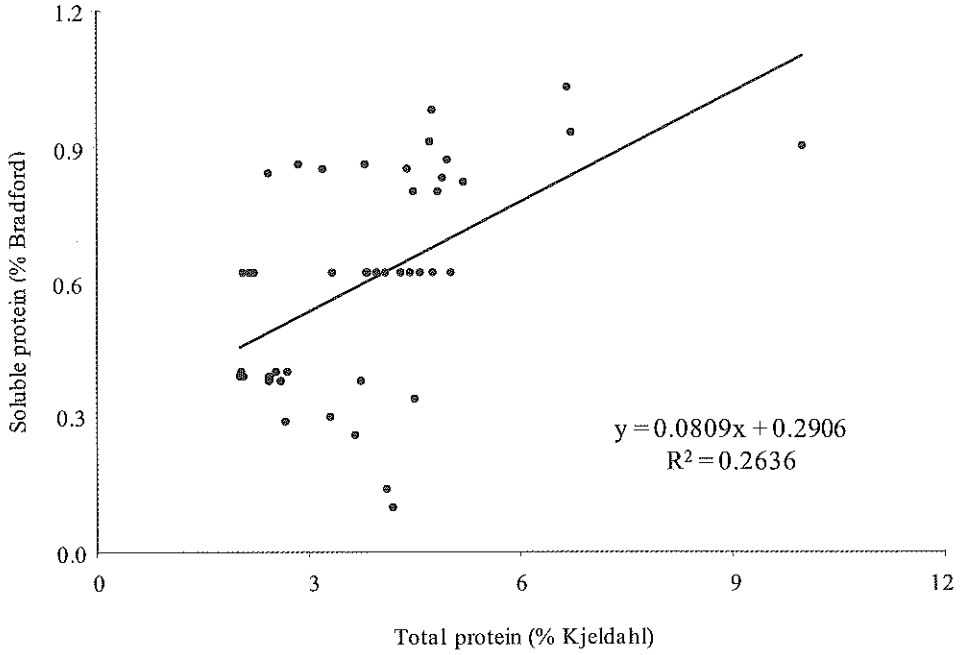

Figure 5. Relationship between TSPC (Bradford method) and crude protein content based on indirect quantification of N content (Kjeldahl's method) based on 45 samples.

Figure 5 illustrates the relationship between TSPC and the crude protein content based on the Kjeldahl's method for the 45 genotypes where N was quantified. This would suggest that the N to protein conversion factor is considerably lower that the conventional factor of 6.25, which is in agreement with reports in the literature (Hock-Hin and Van Den, 1996). The relationship between the two quantification methods was not very strong. The use of protease inhibitors in several samples resulted in similar TSPC, suggesting that proteases activity was not relevant in this type of assay. The Bradford method is a simple, reliable and not an expensive approach to quantify a large number of samples. The amount of individual amino acids in some of the samples analyzed (through HPLC) would allow

quantifying the total protein content (soluble and non-soluble), provide an idea of their nutritional quality and estimate the amount of proteins not accounted for by the Bradford method.

Tolerance to Post-harvest Physiological Deterioration

Cassava roots have a very short shelf life due to a process known as post-harvest physiological deterioration (PPD). PPD rapidly renders the roots unpalatable and unmarketable (Han *et al.*, 2001; Reilly *et al.*, 2003; 2007). Consequently, cassava roots need to be consumed or processed soon after harvesting (Van Oirschot *et al.*, 2000). The short shelf-life of the roots severely limits the marketing options by increasing the likelihood of losses and thus the overall marketing costs. In addition, the access to urban markets and processing facilities is restricted to production sites that are relatively close to them. PPD begins with vascular streaking, which is a blue-black discoloration of the xylem parenchyma, followed by general discoloration of the storage parenchyma. Occlusions and tyloses have also been observed (Rickard *et al.*, 1979). Five to seven days later, microbial activity causes further deterioration. Additionally, respiration increases and starch is hydrolyzed (Hirose *et al.*, 1984; Uritani *et al.*, 1984). The processes involved in PPD resemble typical changes associated with the plant's response to wounding, and triggers a cascade of biochemical reactions in which reactive oxygen species are central. Specific genes involved in PPD have been identified and characterized, and their expressions evaluated (Reilly *et al.*, 2001; Cortés *et al.*, 2002).

Several genotypes considered as potential sources of tolerance to PPD were evaluated (**Table 9**). Results from this evaluation are presented in **Table 10**. Three genotypes did not show any symptoms of PPD even after 40 days of storage (GM 905-66, AM 206-5 and WAXY 4). In addition, mutagenized genotypes, and one of the backcrosses from the interspecific cross with *M. walkerae*, also showed low values of PPD. This is a remarkable finding where many different sources of tolerance to PPD seem to have been discovered; this highlights the importance of aggressive and systematic screening of germplasm for different traits.

Genetic Transformation

The genetic engineering of industrial cassava varieties to produce waxy starch via anti-sense down-regulation of the GBSSI gene has been reported (Salehuzzaman *et al.*, 1993; Munyikwa *et al.*, 1997). GBSSI is the predominant starch synthase gene that catalyses the conversion of ADP-glucose into amylose. The isolation of a full-length GBSSI cDNA clone, construction of sense- and anti-sense transformation cassettes, and their insertion into the genome have been described (CIAT, 2003). Two genetic constructions with the GBSSI gene in anti-sense and sense orientation in the vector pCAMBIA 1305.2 were made to achieve silencing of the gene. The constructs were initially introduced into friable embryogenic callus (FEC) of the model transformation genotype 'MNig 11', from Nigeria, via *Agrobacterium tumefaciens*. Results of GUS transitory assay revealed a successful incorporation of the gene. Transformation of cassava accession MCol 2215 from Colombia was also attempted. Many different transgenic events have been successfully conducted in cassava (Taylor *et al.*, 2004).

High Capacity Root Quality Laboratory

Each year, cassava breeding projects around the world produce thousands of new genotypes. Early stages of selection eliminate a large proportion of these new genotypes without analyzing the quality of their starches or their nutritional properties (Kawano *et al.*, 1998; Jennings and Iglesias, 2002; CIAT, 2003; Kawano, 2003). It is possible, therefore, that along with the eliminated clones, valuable starch or nutritional quality traits have also been discarded. One of the problems, as explained above, is that starch mutants in cassava roots are not as readily identifiable as those in the kernels of cereals.

Table 9. Description of germplasm analyzed for PPD resistance in this study.

Genotype	Observations
CM 523-7 MCol 1505	Susceptible checks
MPer 183	Tolerant check
2G15-1 5G108-4	M_2 genotypes derived from a population mutagenized with gamma rays.
CW 429-1	Inter-specific cross (F_1) with *M. walkerae*
BC 284-42 BC 284-49	First backcrosses to elite clone MTai 8
BC 289-30	First backcross to elite clone SM909-258
CB 7-9 CB 44-15 GM 905-66 MBra 253 MCol 2436	Yellow roots/high carotenes. GM 905-66 is the genotype whose roots were found without PPD symptoms two months after harvest. CB genotypes were from Brazil and MCol 2436 and MBra 253 are landraces from the germplasm collection
AM 206-5	Original source of amylose-free starch
WAXY 2 WAXY 3 WAXY 4 WAXY 5 WAXY 6 WAXY 7	Genotypes with amylose-free starch derived from AM 206-5

Some of the approaches described above (TILLING system for mutation breeding or the iodine test) are specifically targeting the identification of known mutations (e.g. waxy starch). However, it is valid to assume that unknown mutations may also be available in cassava. A common need of many of the strategies described in this paper is for the availability of a high capacity root quality analysis laboratory to screen large numbers of samples (>15,000/year) in search of those genotypes with novel pasting properties of starch or enhanced nutritional value. CIAT is developing, jointly with the Universidad Nacional de Colombia, a laboratory that will be able to generate thousands of amylograms per year using a battery of rapid viscoanalyzers, Brabender, DSC, and other standard equipment and protocols. For crude protein content, the standardization of curves for the use of Near Infrared Analyzers (NIRs) greatly facilitates the breeding work.

Table 10. Post-harvest physiological deterioration (PPD) quantified at 5, 10, 20 and 40 days after harvest. Average total carotenoids content (TCC) and dry matter content (DMC) and number of roots used for each evaluation are also shown.

Clone	PPD (%)					TCC	DMC	Number of roots			
Variable	5	10	20	40	**Mean**	(ug/g)	(%)	5	10	20	40
CM 523-7	27.1	40.7	57.1	64.1	**47.2**	0.4	44.8	10	10	8	5
MCol 1505	25.7	31.6	71.6	66.4	**48.8**	0.7	40.1	10	10	10	9
MPer 183	5.4	4.0	5.3	9.2	**6.0**	0.5	41.3	10	10	10	5
2G15-1	0.5	0.0	0.0	6.9	**1.9**	1.0	44.0	10	10	10	10
5G108-4	2.9	3.7	7.1	7.3	**5.3**	0.7	45.4	10	10	10	9
CW 429-1	12.5	20.7	23.2	18.6	**18.7**	0.6	37.2	7	6	4	3
BC 284-42	16.8	14.1	16.0	n.a.	**15.6**	0.7	40.5	3	5	9	n.a.
BC 284-49	4.7	4.8	23.3	n.a.	**10.9**	2.5	27.4	4	6	6	n.a.
BC 289-30	0.0	1.0	0.0	0.0	**0.3**	0.5	34.5	10	5	10	10
CB 7-9	3.6	10.9	0.0	1.0	**3.8**	10.2	35.8	10	10	10	6
CB 44-15	0.5	0.0	1.0	1.0	**0.6**	11.5	29.5	10	10	7	5
GM 905-66	0.0	0.0	0.0	0.0	**0.0**	11.1	38.3	2	2	2	2
MBra 253	1.5	0.0	2.9	0.0	**1.1**	9.5	42.0	10	10	4	4
MCol 2436	0.0	26.3	38.9	n.a.	**21.7**	9.1	34.6	10	10	5	n.a.
AM 206-5	0.0	0.0	0.0	0.0	**0.0**	0.7	38.5	10	10	10	7
WAXY 2	3.7	8.0	3.6	3.6	**4.7**	0.6	35.6	10	10	8	8
WAXY 3	0.2	0.0	3.7	6.8	**2.7**	0.5	42.2	10	10	7	4
WAXY 4	0.0	0.0	0.0	0.0	**0.0**	0.6	36.2	10	10	10	4
WAXY 5	0.0	0.0	4.1	0.0	**1.0**	0.9	40.2	10	10	9	6
WAXY 6	3.4	1.4	4.7	2.2	**2.9**	0.5	36.1	10	9	10	9
WAXY 7	18.2	31.1	30.4	30.4	**27.5**	1.0	40.0	10	9	8	8
Average	**6.0**	**9.4**	**13.9**	**12.1**	**10.5**	**2.8**	**39.9**	**8.9**	**8.7**	**8.0**	**6.3**

CONCLUSIONS

Cassava is an important crop in the agriculture of many tropical and subtropical countries. It remains one of the most relevant commodities for subsistence farming as food security, and it is acquiring an increasing role in rural development as raw material for many processing pathways. Starch production from cassava roots and the use of dried chips for animal feeding are clearly the most important examples of industrial uses of cassava, particularly in Asia (Chutharatkul, 2005). To maintain this trend and make cassava even more competitive, several approaches have to be taken simultaneously: increased yields and reduced costs of production; wider uses of cassava products; and increased emphasis on the search for value-added traits/products.

Increased productivity has been achieved successfully through the development of high-yielding clones, improved fertilization and soil conservation approaches, and/or better

management of the planting materials (Kawano, 2003; Chutharatkul, 2005; Hoang Kim *et al.*, 2005; Howeler, 2005). Mechanization is gradually being incorporated in the production system with the development of machinery specifically designed for cassava in order to reduce production costs. New strategies are currently under way in the search for tolerance to herbicides, which offers great promises. Tolerance to herbicides allows for minimum tillage practices, which in turn will maximize water and fertilizer use efficiency. The exploitation of foliage for animal feeding opens a new avenue of uses for cassava. All these approaches have had a remarkable impact on the livelihood of millions of cassava farmers in different tropical and subtropical regions of the world.

This paper described a drastic change in the goals of the cassava breeding project at CIAT towards the production and identification of high-value clones. Success in this regard has been achieved through the identification of starch mutations, unusual plant architecture phenotypes, tolerance to post-harvest physiological deterioration, and increased nutritional value. These are traits what were only in the wish list a few years ago (Ceballos *et al.*, 2006a).

The access to these high-value traits will further contribute to poverty alleviation (a chronic and typical problem of many of the marginal agriculture land where cassava is one of the few crops that can be grown) and rural development (cassava roots need to be processed near the fields where they are harvested). Implicit in this new strategy for cassava genetic improvement is the acknowledgement that these high-value cassava clones will target specific needs of the industry and, therefore, an all-purpose variety will no longer be feasible. For instance, high-carotene, high-protein cassava clones are ideal for the feed industry (as well as for human consumption) but they will present additional problems to the starch industry.

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